Functional Neuroanatomy

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Chapter 1

Electron Microscopy of Golgi-Impregnated Neurons

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Introduction

This chapter will describe and discuss a combined Golgi–electron microscopy (EM) technique that successfully preserves gross morphology and ultrastructure, especially the synaptology, of identified neurons. In addition, various combinations of fixation, chromation and impregnation useful for insect and vertebrate nervous tissue are described. The Appendix lists schedules for fixation and chromation, giving impregnation times.

The most pleasant experience for a neuroanatomist is to know that certain profiles seen in the EM belong to a neuron already seen in the light microscope (LM), particularly in the case of intricate cells whose perikarya and largest processes only are identifiable in thin sections. The present Golgi–EM modification permits synaptic and morphometric analysis of all parts of a nerve cell.

Golgi-stained neurons have been observed in LM and EM by many investigators working on vertebrates (Stell 1964; Blackstad 1965), and insects (Trujillo-Cenóz and Melamed 1970; Strausfeld 1973; Strausfeld and Campos-Ortega 1973; Campos-Ortega and Strausfeld 1973; Ribi 1975), all of whom devised methods for re-embedding Golgi-stained neurons. Other examples of Golgi–EM studies are: Kolb (1970), LeVay (1973), Pinching and Brooke (1973), West (1976), Parnavelas et al. (1977), Peters and Fairén (1978), Peters et al. (1979) and Fairén and Valverde (1980).

Ideal sections containing neurons of special interest, impregnated against a clear background, were removed for EM examination before the embedding medium (usually Permount) had set. The sections were washed in xylene and re-embedded in araldite for ultrathin sectioning. Additional contrast was rarely (or sparingly) applied, since aqueous treatment risks removing the silver chromate precipitate that fills impregnated cells. It is not surprising that the general quality of ultrathin sections has not been satisfactory. In particular, tissue was poorly preserved after rough and unphysiological treatment and suitable only for LM. The omission of OsO_4, uranyl ace-

tate and lead citrate was generally disadvantageous, so that a specific modification of existing Golgi–EM techniques was called for.

The development of aldehyde-based fixatives with physiologic osmolarities and ionic concentrations has refined the method so as to allow combined LM and EM examination of impregnated neurons in the context of their immediate surroundings of unimpregnated neighbours.

Attempts have also been made, especially by vertebrate neuroanatomists, to preserve the cytological details of the impregnated neurons by de-impregnation of normal Golgi-stained tissue prior to embedding for EM. Blackstad (1970) used either sodium sulfite or ammonium nitrate to remove silver chromate deposits, while Ramón-Moliner and Ferrari (1972) substituted silver chromate by lead nitrate. More recently, Ramón-Moliner and Ferrari (1976) have improved their procedure by using lead lactate to produce lead chromate. The initial results were not encouraging, as the preservation of the tissue was poor and the substitution difficult to control.

Another method proposed by Blackstad (1975a, b) consisted of reducing silver chromate to metallic silver by putting thick sections (20–30 μm) containing silver-impregnated cells in glycerol and exposing them to UV-irradiation. Afterwards excess silver chromate was removed with sodium thiosulfate. Only scattered silver particles remained in the neuron originally impregnated with silver chromate. Apart from being difficult to control, the usefulness of this method is limited: sections thicker than 30 μm limit penetration of UV-irradiation.

Fairén et al. (1977) successfully substituted part of the silver chromate deposit with metallic gold prior to removing the remaining silver chromate with sodium thiosulfate. This technique leaves only a minimum amount of gold deposit in the previously impregnated structures. The fine structure of the impregnated fibre is well-preserved, allowing recognition of synapses in the impregnated neuron.

To overcome the complexity of de-impregnation and substitution methods, a sparser precipitation can be obtained using short impregnation times (Scott and Guillery 1974).

The procedure described here is a Golgi–EM technique initially employed for insects (Ribi 1976a) and modified for vertebrate nervous tissue (Ribi and Berg 1980). In this method, silver chromate precipitation is stopped before the cell processes are completely filled, resulting in an incomplete impregnation. This can be achieved by weak chromate and silver solutions and shorter impregnation times, providing a simple, rapid, and reliable Golgi–EM method. Impregnated neurons can be studied in thick sections; they have improved preservation of ultrastructure and are easier to thin-section.

General Description of the Procedure

Insects.

Golgi–EM investigations were carried out on the optic lobes of bees and flies. Insects were immobilized by cooling to 4 °C for a few minutes before the head capsules were opened to allow the fixative to directly contact the brains. Opening certain parts of the head capsule helps local impregnation. Removing the cornea results in impregnated neurons in the first optic neuropil. Removing ommatidia results in impregnated neurons in the first to third optic neuropils and in the midbrain. To resolve neurons extending from the head to the body, openings are made ventrally in the thorax to allow diffusion of silver nitrate (Strausfeld 1980).

Vertebrates

Golgi–EM investigations were mainly carried out on the cerebellar and cerebral cortex of mice and rats. Animals, aged 4 to 5 months, were anaesthetized by intraperitoneal injection of 35% chloral hydrate at a dosage of 0.1 ml/100 g body weight (Palay and Chan-Palay 1974), artificially respired with 95% oxygen and 5% carbon dioxide, and then perfused through the aorta using a two-step aldehyde solution [in 0.12 M phosphate buffer with 0.02 mM $CaCl_2$ (pH 7.35) at 20 °C as recommended by Palay and Chan-Palay (1974)]. Initially 150 ml (1% formaldehyde, 1.25% glutaraldehyde) was used for each animal. After perfusion the heads were removed and stored overnight at 4 °C in a fresh perfusion solution.

The following day the brains were removed from the skulls and divided into pieces not larger than 125 mm^3 and rinsed in three changes of buffer before further treatment.

Fixation

In general, any aldehyde fixation suitable for EM can be used before chromation. The use of aldehyde fixative combined with phosphate buffer (Millonig 1961) gives good results on the above-mentioned species. Modifications of fixatives and buffers may favour impregnation of certain brain areas in different species (see Appendix).

Chromation and silver impregnation can be carried out in the dark or light without noticeable differences in quality of impregnation. Insect and vertebrate nervous tissue shown here was, however, processed in the dark at 4 °C.

The fixation time depends mainly on the tissue size and temperature. Insects the size of *Drosophila* are fixed for at least 3–4 h at 4 °C, whereas after

perfusion vertebrate tissue was left in the final fixative overnight at 4 °C. The pH of the fixative is less critical than is its osmolarity, which should be physiologically appropriate. Fixatives were buffered to a pH value of between 7.2 and 7.4. Tissue was later washed in several changes of buffer to remove excess fixative and then stored in buffer at room temperature prior to osmication and chromation.

Osmication and Chromation

Since osmium tetroxide penetrates slowly, only a small zone of tissue near the surface will be secondarily fixed unless tissue is cut into small pieces. The normal concentration is 1%–2% aqueous or buffered OsO_4. Fixation is for 1–2 h, depending on the volume of the tissue. The duration and concentration of chromation can, however, be varied between 2 and 5 days without resulting in ultrastructural change or differences in the quality of silver impregnation.

Four different procedures gave equally good results:

1. Chromation is incorporated into initial fixation.
2. Chromation follows initial fixation, preceding osmication.
3. Fixation is followed by OsO_4 before chromation.
4. Chromation is incorporated with osmication (the "Golgi-rapid" method).

The following describes the third procedure. Chromation for 2–12 h (at 4 °C) in 1% $K_2Cr_2O_7$ solution (buffered or unbuffered) follows osmication (buffered 2% OsO_4). Tissue is then rinsed in phosphate buffer and, finally, rinsed in double-distilled water. Prolonged chromation does not increase silver impregnation (Strausfeld 1980).

Silver Impregnation

Following chromation, tissue is briefly rinsed in distilled water and then transferred through several changes of 0.5% silver nitrate until there is no precipitate from the tissue surface. To obtain incomplete impregnation, the immersion time is short and the silver concentration low.

Silver impregnation in 0.5% $AgNO_3$ (pH 6.9) is at 4 °C for 0.5 h to (maximally) 4 h in the dark. The tissue blocks were lightly agitated approximately every 15 min to facilitate access of the solution to all sides of the tissue.

After silver impregnation the tissue is washed several times in double-distilled water, dehydrated through ethanols, from 30% to absolute, and washed twice in propylene oxide and finally embedded in epoxy resin in the usual way for routine EM.

Thick Sectioning

The purpose of a planned Golgi–EM study is to analyse individual neurons, axons or dendrites. In such cases it is essential to first cut 20–80 μm thick sections, since searching for the desired structures by EM would require hundreds of ultrathin sections. Thick sections are inspected in the LM before re-embedding and proceeding further for EM.

The Golgi–EM technique uses a mixture of "soft araldite" as the embedding medium. This allows easy cutting of up to 100-μm-thick sections. In the early days of Golgi–EM, thick sections were first mounted in Permount between glass slide and coverslip. Removing a suitable section meant immersing the whole slide in xylene to dissolve the Permount. This procedure (contact between araldite, Permount and xylene) changed the cutting quality of the resin, making ultrathin sectioning difficult.

The following technique considers both requirements: easy handling and cutting of thick, semithin and ultrathin sections. Hard plastic sections (normal EM epoxy mixture) 20–80 μm thick are cut on a sliding microtome equipped with a heavy steel knife or with sturdy razor blades (Schick Injector blades) mounted in a special holder (Jung). The block face is softened during sectioning by illuminating it with an infrared lamp (Philips IR 230–250 V/250 W). Alternatively, a small soldering iron mounted a few millimeters in front of the block face can be used to warm up its surface. The correct degree of softening can be regulated by the cutting speed, or the distance and temperature of the soldering iron or IR bulb.

Instead of thick sections being mounted under Permount between glass slide and coverslip, they are placed in liquid resin between acetate sheets. The advantage of this is that single sections can easily be cut out and stripped of acetate foil without damaging the tissue, or loosening neighbouring sections (Ribi 1978). The cutting quality of the section also remains stable. Thick sections are mounted as follows:

A piece of 0.1-mm-thick acetate foil is attached to a glass slide with double-sided Scotch tape. Thick sections are glued in rows on the acetate foil with a little resin. To flatten the sections the slide is placed on a hot plate (60 °C) for a few minutes. A second piece of acetate foil is then put on the top of the sections. Another glass slide is then weighted down on top of it. After 48 h polymerization of 60 °C the top slide is removed and the top acetate foil peeled off from the sections (Fig. 1).

Re-embedding

Thick sections mounted on the lower acetate sheet are examined under the LM, and appropriately impregnated cells photographed, dissected out with a microscalpel, and remounted in a flat silicon mould (Fig. 1) for an ultrathin cross- or longitudinal-section series.

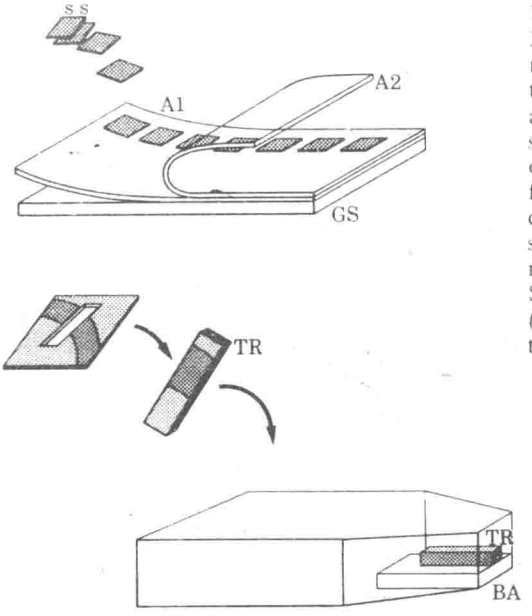


Fig. 1. Re-embedding procedure. 25–80 μm thick sections (*S*) are mounted with a drop of araldite between two sheets of a 0.1 mm thick acetate foil (*A1* and *A2*) on a glass slide *GS*. After polymerization in the oven for 24 h at 60 °C the top acetate foil (*A2*) can be removed. Sections containing neurons of interest are dissected out and the target neuron (*TR*) re-embedded in a flat silicon mould. Small pieces of hardened araldite (*BA*) are first put beneath the section to prevent it from tilting or sinking

Semithin Sectioning

The conventional technique of cutting, staining and examining semithin sections (0.5–2 μm thick) is used on Golgi–EM material to control trimming and to aid orientation before making ultrathin sections.

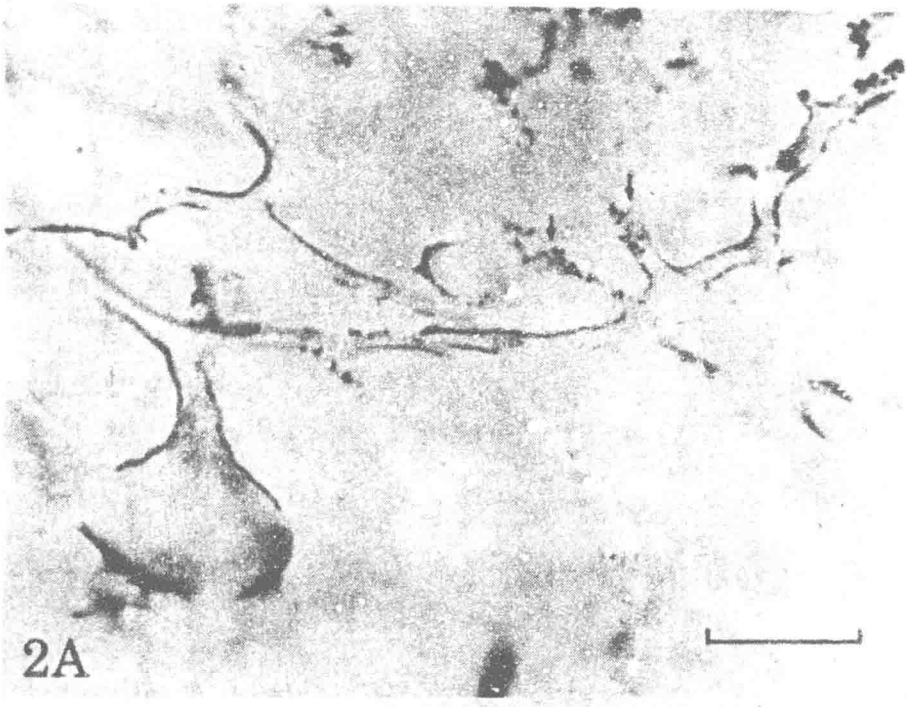
Semithin sections are stained with 1% toluidine blue in 1% borax. Mounting sections in high viscosity immersion oil under a coverslip prevents rapid bleaching.

Ultrathin Sectioning

Trimming and ultramicrotomy of Golgi–EM treated material is carried out conventionally after the block face has been trimmed as much as possible to minimize the search area.

Ultrathin sections are collected on slot grids, coated with 0.25% Formvar and stained for 30 min with a saturated uranyl acetate solution made up in methanol. They are then stained by a modified lead citrate procedure (Venable and Coggeshall 1965) for 20 min.

Fig. 2. A A Purkinje cell of the mouse cerebellum showing the entire inner membrane ensheathed by a thin layer of precipitate. Even small dendritic spines (*small arrows*) contain the precipitate. **B** Pyramidal cell of the mouse cortex. Dendrites with numerous spines and the single axon are lightly impregnated. The cell body appears darker. *Small arrows* indicate dendritic spines. *Ax* axon; *D* dendrite. Bars **A, B** = 20 μm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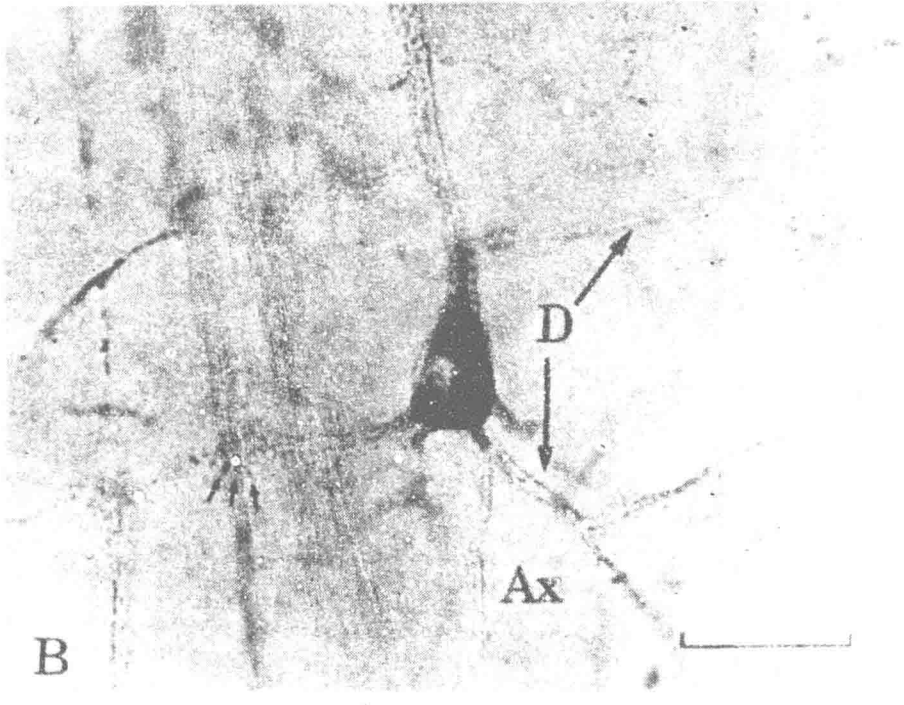
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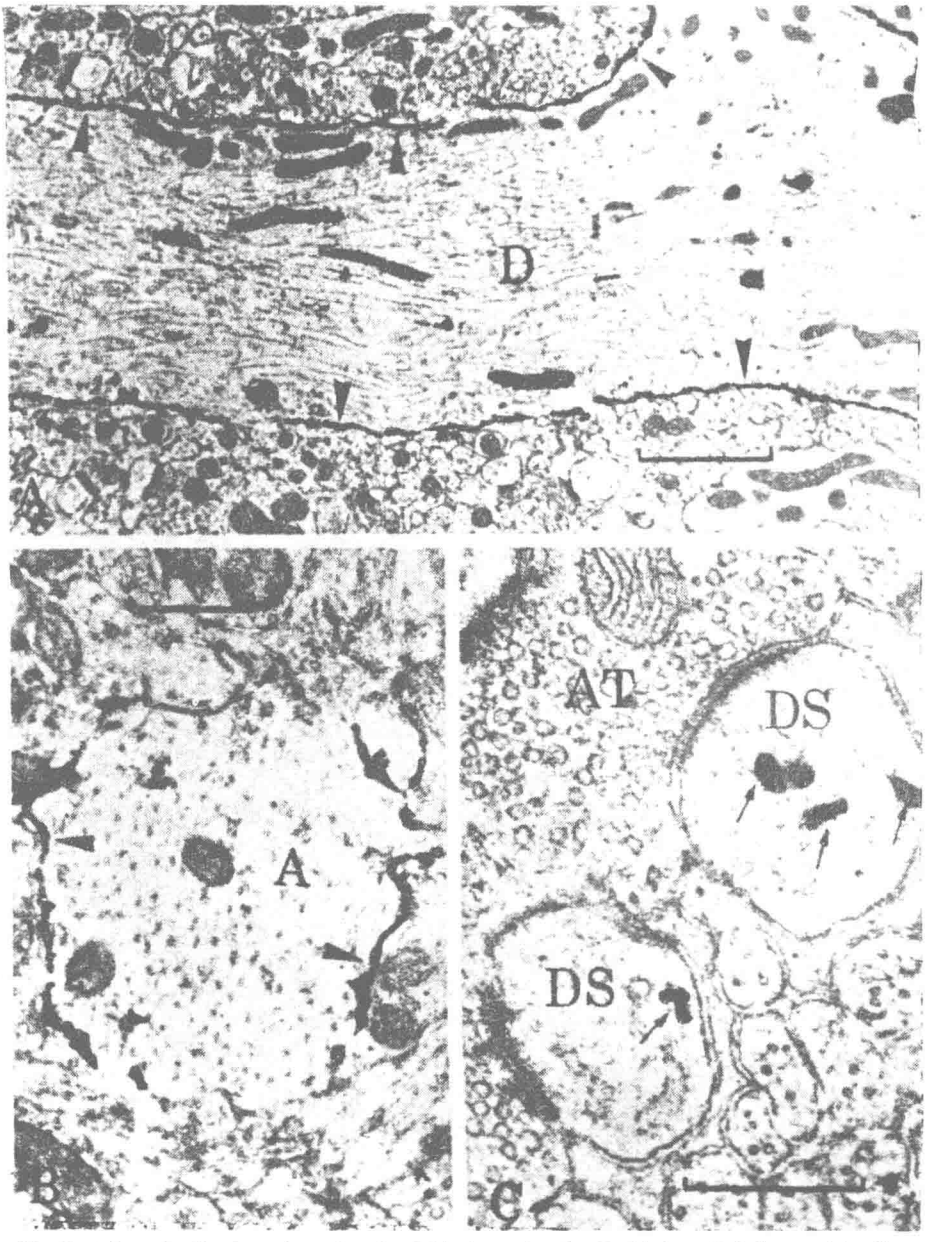

Fig. 3. **A** Longitudinal section of a dendritic branch of a Purkinje cell delineated by fine silver chromate precipitates along the cell membrane. *D* dendrite; *arrowheads* silver precipitate. **B** Cross section through an axonal (*A*) branch of a pyramidal cell. Like the dendrites of cerebellar neurons, the cortical pyramidal cells are delineated by a fine silver precipitate (*arrowhead*). **C** Two dendritic spines in cross section with silver chromate particles located on the spine apparatus (*arrows*). Pre- and postsynaptic sites are well-preserved. *AT* axon terminal; *DS* dendritic spine. Bar **A** = 2 μm, **B, C** = 0.5 μm